

MITOCHONDRIAL ENZYMES IN MANGO FRUIT DURING RIPENING

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Abstract—Mitochondria isolated from immature (developing), mature (unripe), and ripe mango pulp actively oxidized the intermediates of the Krebs cycle. The oxidation of citrate, oxoglutarate, succinate and malate by both unripe and ripe fruit mitochondria was several fold greater than that by mitochondria from immature fruit. The levels of malic dehydrogenase and succinic dehydrogenase increased with the onset of ripening, whereas the level of citrate synthase increased several fold on maturation but decreased six-fold on ripening. Isocitrate dehydrogenase and malic enzyme were very high in the immature fruit but after a sudden decrease in the matured fruit showed a considerable rise thereafter. The ratio of the activities of isocitrate lyase to isocitrate dehydrogenase is considerably higher in the immature fruit and greatest in the unripe (mature) fruit. This, together with a higher concentration of glyoxylate at these stages, indicate the operation of the glyoxylate bypass. Oxidized and reduced forms of pyridine nucleotides were estimated.

INTRODUCTION

STUDIES on the biochemistry of fruit ripening have received considerable attention in recent years.¹⁻³ Major interest has been directed at finding out the cause(s) of the sudden increase in respiration in climacteric fruits.³⁻⁶ Preclimacteric fruits contain considerable amounts of organic acids which may serve as preferred substrates for oxidation by mitochondria and hence result in a high rate of oxidative activity of the fruits after harvest, as is evident from the reports available on apple,^{7,8} banana⁹ and tomato.¹⁰

In ripening mango there is a considerable increase in respiration¹¹ as well as in the enzymes connected with oxidative processes,^{11,12} glycolytic sequence,^{12,13} and the hexose

¹ BURG, S. P. (1962) *Ann. Rev. Plant Physiol.* **13**, 265.

² SPENCER, M. (1965) in *Plant Biochemistry* (BONNER, J. and VARNER, J. E., eds.) pp. 793-825. Academic Press, New York.

³ HULME, A. C. (1970) in *The Biochemistry of Fruits and Their Product* (HULME, A. C., ed.) Vol. I. Academic Press, New York.

⁴ BAIN, J. M. and MERCER, F. V. (1964) *Australian J. Biol. Sci.* **17**, 78.

⁵ RHODES, M. J. C. and WOOLVERTON, L. S. C. (1967) *Phytochemistry* **6**, 1.

⁶ SACHER, J. A. (1966) *Plant Physiol.* **41**, 707.

⁷ PEARSON, J. A. and ROBERTSON, R. N. (1954) *Australian J. Biol. Sci.* **7**, 1.

⁸ NEAL, C. E. and HULME, A. C. (1958) *J. Exp. Botany* **9**, 142.

⁹ HAARD, N. F. and HULTIN, H. O. (1968) *Anal. Biochem.* **24**, 299.

¹⁰ DICKINSON, D. B. and HANSON, J. B. (1965) *Plant Physiol.* **40**, 161.

¹¹ MATTOO, A. K., MODI, V. V. and REDDY, V. V. R. (1968) *Indian J. Biochem.* **5**, 111.

¹² MATTOO, A. K. (1969) Some aspects of metabolic changes involved in ripening mangoes. Ph.D. Thesis. M.S. Univ. Baroda, India.

¹³ GEETHA GHAI (1972) Carbohydrate metabolism in ripening mangoes. Ph.D. Thesis. M.S. Univ. Baroda, India.

monophosphate shunt.¹⁴ During these studies no attempts were made to obtain data on immature (developing) fruit. We have now studied mitochondrial oxidation of various substrates and also the levels of Krebs cycle enzymes in mango at immature (developing), mature (unripe), and ripe stages.

RESULTS AND DISCUSSION

Mitochondrial oxidations

Mitochondria isolated from immature, unripe and ripe mango pulps are active and oxidize citrate, oxoglutarate, succinate and malate effectively (Table 1). Oxidizing capacity of immature fruit mitochondria is much less than from unripe and ripe fruit. The data indicate that mitochondria in mango are active at all stages of ripening and development, and that during ripening this activity increases considerably. Earlier, increase in the mitochondrial oxidation of pyruvate with ripening was reported by Mattoo *et al.*¹¹ in mangoes. This trend in increased oxidation rate during ripening seems to follow that reported for apple,^{7,8} banana,¹⁵ lemon¹⁶ and tomato.^{10,17}

Levels of Krebs cycle enzymes

The overall changes in the mitochondrial oxidations (Table 1) suggest that the enzymes connected with the Krebs cycle may be synthesized or activated during the ripening process, and the levels of these enzymes were therefore studied (Table 2). The levels of the enzymes of the Krebs cycle increase considerably during ripening. Citrate accumulation during maturation of the mango (Baqui, Mattoo and Modi, unpublished results) is accompanied by higher levels of citrate synthase in the immature and unripe fruit and its decrease six-fold during ripening (Table 2). Decrease in the content of citrate and malate during ripening¹⁴ may be related to the decreased citrate synthase (Table 2), and increased levels of the citrate cleavage enzyme¹⁸ and those of isocitrate dehydrogenase, succinic dehydrogenase and malic dehydrogenase (Table 2) during ripening, showing that citrate and malate catabolism is an important phenomenon of the mango with respect to increased respiration during ripening.

TABLE 1. OXIDATION OF SOME KREBS CYCLE INTERMEDIATES BY MITOCHONDRIA FROM IMMATURE (DEVELOPING), MATURE (UNRIPE) AND RIPE MANGO

Substrate*	$\mu\text{l O}_2$ consumed/hr/mg dry wt mitochondria		
	Immature	Mature	Ripe
Citrate	11.7	37.2	60.8
Oxoglutarate	15.3	29.4	48.3
Succinate	18.1	24.0	58.9
Malate	24	30.6	70.3

* Each substrate concentration was 27 mM.

The level of isocitrate lyase is remarkable in the preharvest fruit; it increases two-fold in the developed and mature (unripe) fruit (Table 2) and decreases three-fold in the ripe

¹⁴ MODI, V. V. and REDDY, V. V. R. (1967) *Indian J. Expt. Biol.* **5**, 233.

¹⁵ HAARD, N. F. and HULTIN, H. O. (1969) *Phytochemistry* **8**, 2149.

¹⁶ BOGIN, E. and ERICKSON, L. C. (1965) *Plant Physiol.* **40**, 566.

¹⁷ KU, H. S., PRATT, H. K., SPURR, A. R. and HARRIS, W. M. (1968) *Plant Physiol.* **43**, 883.

¹⁸ MATTOO, A. K. and MODI, V. V. (1970) *Biochem. Biophys. Res. Comm.* **39**, 895.

fruit. The ratio of the activities of isocitrate lyase to isocitrate dehydrogenase changes from 3.2 in the immature fruit to 15 in the unripe (matured) fruit and to 0.54 in the ripe mango fruit (Table 2). In addition we have found that the concentration of glyoxylate, one of the products of isocitrate lyase, increases from 9 $\mu\text{mol/g}$ fr. wt in immature to 14.5 $\mu\text{mol/g}$ fr. wt in the unripe and falls thereafter to 5 $\mu\text{mol/g}$ fr. wt in the partly ripe and to 2.5 $\mu\text{mol/g}$ fr. wt in the ripe fruit. The higher amounts of glyoxylate and malate¹⁴ (the product of malate synthetase), and higher ratio of the activities of isocitrate lyase to isocitrate dehydrogenase all indicate the operation of the glyoxylate bypass in the developing (immature) and preclimacteric mango fruit, possibly resulting in the conservation of carbon at these stages. Incipient ripening is accompanied by low levels of malate and glyoxylate and very low ratios of isocitrate lyase to isocitrate dehydrogenase activity. Isocitritase in ripening pear fruit was reported by Meynhardt *et al.*¹⁹

The increase of levels of succinic dehydrogenase and malic dehydrogenase between the immature to the mature stage is dramatic (Table 2). In contrast, and surprisingly, the levels of both isocitrate dehydrogenase and malic enzyme which are very high in the immature fruit, show a sharp decline in the mature fruit with a rapid increase in the ripe fruit which is suggestive of the presence of a regulatory control of these enzymes.

TABLE 2. ACTIVITY OF SOME KREBS CYCLE ENZYMES, ISOCITRATE LYASE AND MALIC ENZYME IN EXTRACTS PREPARED FROM DEVELOPING (IMMATURE), UNRIPE (MATURE) AND RIPE MANGO PULP

	Enzyme activity (units/mg protein)		
	Immature	Mature	Ripe
Citrate synthase	10.9	25.2	4.22
Isocitrate dehydrogenase	18.3	8.18	74
Malic dehydrogenase	5.0	28	54
Succinic dehydrogenase	6.28	11.7	13.3
Malic enzyme	16.2	8.1	35
Isocitrate lyase	58.4	120	40.6

Regulation of malic dehydrogenase and malic enzyme in other fruits have been reported.^{3,20} These enzymes have been shown to possess regulatory properties which may have implications in the ripening process. Also, these enzymes exist in multiple forms in plant tissues.²¹⁻²⁴ On subjecting soluble extracts from unripe and ripe mango pulp to gel electrophoresis only one band with respect to malic enzyme in both the extracts was detectable, whereas the enzyme bands with respect to the malic dehydrogenase were two in the unripe fruit extract and three in the ripe fruit extract. The enzyme activity bands were more intense with ripe fruit extract. Appearance of a new band of malate dehydrogenase in the ripe fruit is suggestive of increased enzyme synthesis during ripening.

¹⁹ MEYNHARDT, J. T. M., MAXIE, E. C. and ROMANI, R. J. (1965) *S. African J. Agric.* **8**, 291.

²⁰ HULME, A. C., JONES, J. D. and WOOLTORTON, L. S. C. (1964) *Phytochemistry* **3**, 173.

²¹ RAGLAND, T. E., CARROLL, E. W. Jr. and PITELKA, L. F. (1972) *Phytochemistry* **11**, 1303.

²² SARKISSIAN, I. V. and MCDANIEL, R. G. (1967) *Proc. Nat. Acad. Sci. U.S.A.* **57**, 1262.

²³ MIFLIN, B. J. (1968) *Biochem. J.* **108**, 49.

²⁴ CLEMENT, R. L. (1970) in *The Biochemistry of Fruits and Their Products* (HULME, A. C., ed.) Vol. I, p. 159. Academic Press, New York.

It was of interest to study the kinetic parameters of these enzymes in the crude cell-free preparations of unripe and ripe fruit pulp. Apparent K_m and V_{max} values were obtained from the Lineweaver-Burk plots of the substrate saturation curves and the calculated apparent kinetic constants are tabulated in Table 3. The apparent K_m values of malic enzyme with respect to both substrates, malate and NADP, did not change on ripening. Similarly the K_m values of malic dehydrogenase with respect to both substrates were the same in the enzymes from unripe and ripe fruit. The apparent K_m value of isocitrate dehydrogenase for DL-isocitrate was 10^{-4} M. Succinic dehydrogenase showed an apparent K_m of 3.5×10^{-3} M with respect to succinate. As the activities of these enzymes in the extracts prepared from unripe mango were very low, we could not compare the substrate saturation curves of these enzymes at these two stages of ripening.

TABLE 3. KINETIC CONSTANTS OF MALIC ENZYME, MALIC DEHYDROGENASE, SUCCINIC DEHYDROGENASE AND ISOCITRATE DEHYDROGENASE FROM MANGO FRUIT

Enzyme	Tissue	Substrate	Apparent K_m (mM)	Apparent V_{max} (units/mg protein)
Malic enzyme	Unripe	L-malate	0.66	10.6
		NADP	0.069	25
	Ripe	L-malate	0.66	37.0
		NADP	0.069	20
Malic dehydrogenase	Unripe	L-malate	0.29	28.9
		NAD	0.040	40
	Ripe	L-malate	0.29	83.3
		NAD	0.040	66
Succinic dehydrogenase	Ripe	Succinate	3.5	13.3
Isocitrate dehydrogenase	Ripe	DL-isocitrate	10.8	74

Levels of pyridine nucleotides

To ascertain whether the low enzyme activity in the immature and unripe fruit was due to the limitation imposed by the concentrations of oxidized and reduced forms of pyridine nucleotides we studied the concentrations of these coenzymes in the immature mango pulp. The concentrations of NAD, NADP, NADH and NADPH in the immature fruit were found to be 26.1, 33.4, 7.67 and 7.99 $\mu\text{g/g}$ fresh pulp respectively and the total pyridine nucleotide content was 75.1 $\mu\text{g/g}$ fresh pulp. Reddy²⁵ had obtained values for these pyridine nucleotides in unripe, partly ripe and ripe mango which were 196, 202 and 191 $\mu\text{g/g}$ fresh pulp respectively. The ratio of NAD plus NADH to NADP plus NADPH changed from approximately 1:1.2 in the immature fruit to approximately 1:1.1 in the unripe stage, to 1.48:1 in the partly ripe stage and to 1:1.3 in the ripe stage. Moreover, the ratio of NAD to NADH does not differ much in the immature and unripe stages as compared to partly ripe and ripe stages of the fruit. In the immature fruit as well as in the unripe one the ratio is 3, in the partly-ripe mango it is 0.75 and at the ripe stage it is 1.75. On the other hand,

²⁵ REDDY, V. V. R. (1968) The mechanism of carotene biosynthesis in mangoes and carrots. Ph.D. Thesis, M.S. Univ. Baroda, India.

the ratio of NADP to NADPH in the immature as well as in the unripe fruit is 1.5, 0.18 in partly ripe and 1.1 in the ripe fruit. These data suggest that although there are much lower levels of pyridine nucleotides in the immature stage, the ratios of NAD plus NADH to NADP plus NADPH, NAD to NADH and NADP to NADPH do not change much from the immature to unripe stages. The dramatic change in their concentrations at the ripening stage could play a significant role in the ripening process since these coenzymes may afford a control of some key enzymes like malic dehydrogenase and isocitrate dehydrogenase. In the immature as well as in the unripe stage, one might expect an increase in the rate of many NADP requiring reactions, since there would be more substrate (NADP) and less product (NADH, NADPH). The product, otherwise, may be utilized in the active reductive synthesis during these stages. Though the oxidizing forms of the coenzymes are appreciable in immature and unripe fruit, the levels of malic dehydrogenase and isocitrate dehydrogenase (Table 2) are not correspondingly high, suggesting that there may be other factors other than the pyridine nucleotides which may control the levels of these enzymes in the fruit at the immature and mature (unripe) stages. Also, it is possible that the synthesis of these enzymes may be very low prior to ripening and/or inhibitors of their activity are present in the fruit at these stages.

TABLE 4. INHIBITION OF MALIC ENZYME AND ISOCITRATE DEHYDROGENASE FROM RIPE MANGO BY DEVELOPING (IMMATURE) FRUIT PULP EXTRACT

	Malic enzyme activity	Inhibition (%)	Isocitrate dehydrogenase activity	Inhibition (%)
Extract from immature control*	0.90	—	1.8	—
Extract from ripe control*	6.29	—	82	—
Extract from ripe + immature†	1.5	79	42	49

* Results are expressed in enzyme units.

† Dialysed unripe and ripe extracts in 1 ml equal proportions were incubated for 10 min at 0° before determining the residual enzyme activity. Ripe and immature controls contained 1 ml of Tris-HCl buffer (pH 7).

Earlier, Mattoo and Modi²⁶ have reported the presence of inhibitors of catalase, peroxidase, amylase and invertase in unripe mango. These inhibitors may control the process of ripening in mango since they found that ethylene, the ripening hormone, inactivated these inhibitors not only from mango but also from banana and papaya. It was of interest, therefore, to see if immature mango contained any such type of inhibitors for malic enzyme and isocitrate dehydrogenase. Immature extracts, freed of phenols and other impurities by treatment with polyvinylpyrrolidone (0.1%) and subsequent dialysis, were mixed with the extracts prepared from the ripe fruit pulp. Proper controls were also made. Results in Table 4 show that the immature extract contains some substance(s), which inhibit the enzymes from ripe fruit. This substance was found to be heat-labile and non-dialysable. It therefore appears that the enzymes may be limited by inhibitors which may be destroyed or inactivated during ripening as was shown for the unripe mango inhibitors.^{26,27}

²⁶ MATTOO, A. K. and MODI, V. V. (1970) *Enzymologia* **39**, 237.

²⁷ MATTOO, A. K. and MODI, V. V. (1969) *Proc. Conf. Trop. Subtrop. Fruit (Lond.)*, 111.

EXPERIMENTAL

Mangoes. (*Mangifera indica* L. var. Alphonso) were obtained from the local market. The various stages of the fruit during ripening were marked by the colour development and appearance. Immature fruit is referred to the stage of mango which is in the development stage, plucked from the tree before it is mature enough for harvesting and will not ripen normally. Unripe fruit refers to the stage of the fruit which is mature with green peel, firm when touched, white pulp and ready to harvest. Partly-ripe refers to the fruit with green to yellow peel, slightly soft and faint yellow pulp and ripe fruits to those which had golden peel, soft when touched and golden yellow pulp. Ripening was usually effected at 25–30° in the laboratory.

Preparation of extracts. The fresh pulp of the fruit was frozen and powdered at –15°. The powder was extracted in 0.1 M Tris-HCl buffer (pH 7.2) containing 0.2 M sucrose and 0.1% polyvinylpyrrolidone (MW 10000) by grinding manually in an unglazed mortar and pestle at 0–5°. During extraction the pH was maintained at 7.2 with 0.1 M NaOH. This fruit extract was centrifuged in cold at 5000 *g* for 15 min. The supernatant was collected and used for enzyme assays. Protein was estimated by the method of Lowry *et al.*²⁸ using serum albumin as the standard.

Isolation of mitochondria. All the operations were carried out at 0–5°. The fruit pulp was extracted (1:2, w/v) in a soln containing sucrose (0.4 M), EDTA (0.6 μ mol) and polyvinylpyrrolidone (0.1%) to obtain a 50% extract. The pH of the extract was maintained at 7.2 with 0.1 M KOH soln during extraction. The homogenate was centrifuged at 3000 *g* for 15 min and the residue discarded. The supernatant was recentrifuged at 15000 *g* for 30 min. Further homogenization of the pellet was done in a glass homogenizer in a soln containing phosphate buffer (0.1 M, pH 7.2), sucrose (0.2 M), ATP (160 μ mol), MgCl₂·6H₂O (250 μ mol) and bovine serum albumin (2 mg/ml) in 50 ml. After centrifuging the residue at 1000 *g* for 5 min the supernatant was decanted and recentrifuged at 15000 *g* for 30 min. The final pellet containing active mitochondria was suspended in the soln described above.

O₂ uptake was determined by the Warburg manometer. The reaction mixture contained NAD (1 μ mol), thiamine pyrophosphate (1 μ mol), cytochrome *c* (6×10^{-5} μ mol), sucrose (100 μ mol), phosphate buffer (40 μ mol, pH 7.2), substrate soln (40 μ mol) and mitochondrial preparation (0.08–0.15 mg dry wt) in a final vol of 1.5 ml. The rates were determined up to 60 min and the values extrapolated to zero time.

Enzyme assays. Malic enzyme [E.C. 1.1.1.40, L-malate: NADP oxidoreductase (decarboxylating)] was assayed as described previously.¹⁴ Malic dehydrogenase [E.C. 1.1.1.37 L-malate: NAD oxidoreductase] was estimated by the method of Ochoa.²⁹ The final assay mixture contained in μ mol: Tris-HCl buffer (pH 9), 100; NAD, 0.5; L-malic acid, 50; and an appropriate amount of enzyme soln in a final vol. of 2 ml. Isocitrate dehydrogenase [E.C. 1.1.1.42, *threo*-DS-isocitrate: NADP oxidoreductase (decarboxylating)] was estimated by the method of Ochoa.³⁰ The final assay mixture contained in μ mol: Tris-HCl buffer (pH 7.8), 75; DS-isocitrate, 6; NADP, 0.5, and an appropriate amount of the enzyme. The final vol. was 2 ml. Change A at 340 nm was followed for 5 min. One unit of the enzyme is that amount which causes a change of 0.01 A/min. Succinic dehydrogenase [E.C. 1.3.99.1 succinate: (acceptor) oxidoreductase] was measured by the method of Slater and Bonner,³¹ using ferricyanide as an artificial electron acceptor. The assay mixture contained in μ mol: Na phosphate buffer (pH 7.2), 150; Na succinate, 10; KCN, K₃Fe(CN)₆, 5; and 0.5 ml aliquot of the enzyme soln in a final vol. of 2.5 ml. The incubation was carried out at 37° for 1 hr and the enzyme reaction was terminated by adding 0.5 ml of 50% TCA at 0°. The A of the TCA supernatants was read at 440 nm. One unit of the enzyme is that amount which reduces 1 μ mol of ferricyanide/hr. Isocitrate lyase (E.C. 4.1.3.1-*threo*-DS-isocitrate glyoxylate-lyase) was estimated by the method of Dixon and Kornberg.³² The standard assay mixture contained in μ mol: K phosphate buffer (pH 7.2), 200; DS-isocitrate, 10; MgCl₂·6H₂O, 1.5; phenylhydrazine hydrochloride, 10; cysteine hydrochloride, 6; and an appropriate aliquot of the enzyme soln. The final vol. was 3 ml. Change in A at 324 nm was followed for 5 min. One unit of enzyme is that amount which causes an increase of 0.01 A/min. Citrate synthase [E.C. 4.1.3.7, Citrate oxalo-acetate-lyase (CoA acetylating)] was estimated by the method of Srere and Kosicki.³³ The standard assay mixture contained, Tris-HCl buffer (pH 7.2), 100 μ mol; oxaloacetic acid, 0.6 μ mol; 0.2 μ mol of acetyl-CoA (Sigma) and an appropriate aliquot of the enzyme soln in a final vol. of 2 ml. Change in A at 233 nm, due to both cleavage of the thiolester bond and utilization of OAA, was followed for 5 min. One unit of the enzyme is that amount which catalyzes the formation of 1 μ mol of citrate/min. Glyoxylate was estimated essentially by the colorimetric method of McFadden and Howes.³⁴

²⁸ LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951) *J. Biol. Chem.* **193**, 265.

²⁹ OCHOA, S. (1955) in *Methods in Enzymology* (COLOWICK, S. P. and KAPLAN, N. O., eds.), Vol. 1, p. 735, Academic Press, New York.

³⁰ OCHOA, S. (1955) in *Methods in Enzymology* (COLOWICK, S. P. and KAPLAN, N. O., eds.), Vol. 1, p. 699, Academic Press, New York.

³¹ SLATER, E. C. and BONNER, W. D. (1952) *Biochem. J.* **52**, 185.

³² DIXON, G. H. and KORNBERG, H. L. (1959) *Biochem. J.* **72**, 3.

³³ SRERE, P. A. and KOSICKI, G. W. (1961) *J. biol. Chem.* **236**, 2557.

³⁴ MCFADDEN, B. A. and HOWES, W. V. (1960) *Anal. Biochem.* **1**, 240.

Extraction and estimation of pyridine nucleotides. The reduced and oxidized nucleotides were extracted and estimated by the method of Caiger *et al.*³⁵ with a modification; NADP was estimated by using glucose-6-phosphate dehydrogenase (Sigma).

The assay system for NADP estimation contained: fruit extract (0.6 ml), Tris-HCl buffer (100 μ mol; pH 7.2), glucose-6-phosphate (10 μ mol), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (1 μ mol), and glucose-6-phosphate dehydrogenase (20 units; Sigma) in a final vol. of 1.5 ml. The assay system for NAD estimation contained: fruit extract (0.6 ml); Tris-HCl buffer (100 μ mol; pH 7.2), glycine (15 mg), NaOH (15 mg), EtOH (0.05 ml) and alcohol dehydrogenase (20 units; Sigma) in a final vol. of 1.5 ml. Formation of NADPH/NADH was followed spectrophotometrically at 340 nm. The assay system for NADH estimation contained fruit extract (0.6 ml), Tris-HCl buffer (100 μ mol; pH 7.2), acetaldehyde (4 μ mol) and alcohol dehydrogenase (Sigma; 1 unit) in a final vol. of 1.5 ml. The assay system for NADPH estimation contained: fruit extract (0.2 ml), Tris-HCl buffer (100 μ mol, pH 7.2); glutathione, (5 μ mol) and glutathione reductase (Sigma; 10 units) in a final vol. of 1.5 ml. Formation of NAD/NADP was followed spectrophotometrically at 340 nm.

Electrophoresis. Polyacrylamide gel electrophoresis of cell-free extracts was carried out by the method of Davis³⁶ with 3 mA/gel at 5° for 90 min. Malic dehydrogenase activity bands were localized on the gels by the method of Brain and McDaniel.³⁷ Malic enzyme activity bands were localized on the gels by replacing MgCl_2 and NAD with MnCl_2 and NADP in the reaction mixture used for detecting malic dehydrogenase.

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³⁵ CAIGER, P., MORTON, R. K., FILSELL, O. H. and JARRETT, I. O. (1962) *Biochem. J.* **85**, 351.

³⁶ DAVIS, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404.

³⁷ BRAIN, G. and MCDANIEL, R. G. (1970) *Biochim. Biophys. Acta* **220**, 410.